Expression and Possible Prognostic Role of MAGE-A4, NY-ESO-1, and HER-2 Antigens in Women with Relapsing Invasive Ductal Beast Cancer: Retrospective Immunohistochemical Study

Daniela Bandić[1], Antonio Juretić[2], Božena Šarčević[3], Viktor Šeparović[3], Mirjana Kujundžić Tiljak[4], Tvrtko Hudolin[5], Giulio C. Spagnoli[6], Dinko Čović[1], Mirko Šamija[1]

[1]Department of Anesthesiology, University Hospital for Tumors, Zagreb, Croatia
[2]Department of Oncology, Zagreb University Hospital Center, Zagreb, Croatia
[3]Department of Pathology, University Hospital for Tumors, Zagreb, Croatia
[4]Department of Medical Statistics, Epidemiology and Medical Informatics, Andrija Štampar School of Public Health, Zagreb University School of Medicine, Zagreb, Croatia
[5]Department of Urology, Zagreb University Hospital Center, Zagreb, Croatia
[6]Department of Surgery, Research Laboratory, University Hospital Basel, Basel, Switzerland

> **Correspondence to:**
Antonio Juretić
Department of Oncology
Zagreb University Hospital Center
Kišpatićeva 12
10000 Zagreb, Croatia
antonio.juretic@zg.htnet.hr

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Aim To evaluate the possible prognostic role of the expression of MAGE-A4 and NY-ESO-1 cancer/testis antigens in women diagnosed with invasive ductal breast cancer and determine the expression of HER-2 antigen.

Methods The expression of MAGE-A4, NY-ESO-1, and HER-2 antigens was evaluated immunohistochemically on archival paraffin-embedded samples of breast cancer tissue from 81 patients. All patients had T1 to T3, N0 to N1, M0 tumors and underwent postoperative radiotherapy and, if indicated, systemic therapy (chemotherapy and hormonal therapy). The antigen expression in women who were disease-free for 5 years of follow up (n = 23) was compared with that in women with either locoregional relapse (n = 30) or bone metastases (n = 28). Patient survival after 10 years of follow up was assessed.

Results The three groups of women were comparable in terms of age, type of operation, tumor size, tumor grade, number of metastatically involved axillary lymph nodes, Nottingham prognostic index (NPI), progesterone receptor (PR) status, and adjuvant hormonal therapy. Estrogen receptors (ER) were positive in 13 women in the 5-year relapse-free group vs 8 in locoregional relapse and 7 in bone metastases group ($P = 0.032$). There were significantly fewer women who received adjuvant chemotherapy in the 5-year relapse-free group than in other two groups (7 vs 23 with locoregional relapse and 25 with bone metastases; $P<0.001$). This group also had a significantly better 10-year survival (14 women vs 1 with locoregional relapse and 1 with bone metastases; $P<0.001$). The three groups did not differ in the NY-ESO-1 or HER-2 expression, but the number of patients expressing MAGE-A4 antigen was significantly lower in the group with locoregional relapse ($P = 0.014$). In all groups, MAGE-A4 antigen expression was associated with the NY-ESO-1 antigen expression ($P = 0.006$), but not with tumor size and grade, number of metastatically involved axillary lymph nodes, or the ER and PR status. MAGE-A4-positive patients had a significantly longer survival than the MAGE-A4-negative patients ($P = 0.046$). This was not observed with NY-ESO-1 and HER-2 antigens.

Conclusion Our results suggest that the MAGE-A4 antigen may be used as a tumor marker of potential prognostic relevance.

Breast cancer is the most common malignancy in women (1). Its clinical course may vary from indolent and slowly progressive to rapidly metastatic disease. Identification of prognostic and predictive factors that reflect the biology of breast cancer is important for the assessment of prognosis and selection of patients who may benefit from adjuvant and/or systemic therapy. The important aspects of prognostic factors suitable for clinical use are their availability, reproducibility, and cost. In routine clinical practice, treatment decisions and selection of treatment modalities for each individual patient are based on the standard prognostic factors, such as age (1,2), menopausal status (3), tumor size (1-4), tumor grade (3-5), steroid-hormone receptor status (1-5), and nodal metastases (1-5).

Variability in clinical course of breast cancer is partly related to tumor cell growth rate and other features, such as invasiveness or metastatic potential. Research in molecular biology has identified genes and their products involved in or associated with the malignant cell transformation and behavior. Moreover, expression of some of these molecules, such as p53 (1,6,7), Ki-67 (7,8), nm23 (1,7), catepsin D (1,7), Ep-CAM (9,10), HER-2 (1,2,6), and urokinase-type plasminogen activator and its inhibitor (1,11), is associated with the patient's prognosis. As it seems that many genes and molecules might be involved in malignant transformation and cell behavior, other additional molecules may also be tested as potential prognostic factors.

The cancer/testis (C/T) genes encode tumor-associated antigens (TAA) found in various tumors of different histological origin, but not in normal tissues other than testis (12,13). Their physiological function is unknown. Peptides derived from these antigens could be used as targets in active immunotherapy. Analysis of the expression of these genes or their products in malignancies could also be of potential diagnostic and/or prognostic relevance (14,15). Therefore, we performed a retrospective analysis of immunohistochemical expression of C/T antigens MAGE-A4 and NY-ESO 1 in women with invasive breast cancer. We also analyzed the expression of HER-2 antigen, because it has a prognostic and predictive role (1,16).

Patients and methods

Patients

Women who had invasive breast cancer in 1995 and underwent postoperative adjuvant radiotherapy were identified from medical documentation available at the Department of Radiotherapy, University Hospital for Tumors, Zagreb, in 2000. Only women with complete medical data and adequate tumor-tissue samples available from archival paraffin-embedded blocks were eligible. Women were included in the study if, at the moment of initial diagnosis, they had breast cancer without distant metastases (pT1-3pN0-1M0) for which they received surgical and adjuvant treatment of radiotherapy and, if indicated, systemic treatment (chemotherapy and hormonal therapy) in our Hospital. Women who received neoadjuvant therapy, had another primary cancer, or received treatment at an institution other than ours were excluded from the study. The final number of women included in the analysis was 81. According to the disease status, there were three groups, as follows: women who had been relapse-free for five years, ie, from 1995 to 2000, those with locoregional relapse, and those with bone metastases. Almost all women with locoregional relapse had undergone repeated surgical and irradiation treatment. Locoregional relapse was defined as the first recognized recurrence of the disease in the chest wall, breast, axilla, or supraclavicular region. Women with bone metastases had all undergone irradiation treatment. Those who were diagnosed with invasive breast cancer in years other than 1995 were included in groups with locoregional and

metastatic disease, so that each group consisted of a similar number of patients.

Data collection

Medical files at the Department of Radiotherapy contained the data on the patients' clinicopathological findings, radiotherapy decisions, and/or treatments. Other data, such as those on the existence and sites of visceral organ metastatic disease or previous or planned systemic treatments of metastatic disease, were often not recorded or available for patients who were referred to the Department for radiotherapy and otherwise followed-up by other oncologists, surgeons, or general practitioners. Often, the day of disease relapse (locoregional or metastatic) could not be precisely determined. The complete data were collected on the date of breast cancer operation, pathological findings, and the type of adjuvant systemic therapy.

Patient survival analysis was performed in 2005. The survival data were also checked against the Croatian National Cancer Registry and rechecked whenever possible by telephone calls to patients presumed to be alive. The study protocol was approved by the Ethics Committee of the Hospital and informed consent was obtained from all patients included in the study.

Clinicopathological features

Clinicopathological and laboratory data included age, year of diagnosis, type of surgery, median time to disease relapse, tumor size, histological grade, axillary node status, Nottingham Prognostic Index (NPI) (17), estrogen and progesterone receptor positivity, administration of adjuvant chemotherapy or of adjuvant tamoxifen, and patient survival (Table 1).

Pathological examination of primary tumors and axillary lymph nodes was performed at the Department of Pathology. For routine histological analysis, the resected tissue was fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The histological grade of tumors was determined according to the method by Elston (18). Tumors were divided into three groups according to tumor size (0.1-2.0 cm as pT1, 2.1-5.0 cm as pT2, and >5.0 cm as pT3) and the ipsilateral axillary lymph node status (negative lymph node as N0, and positive lymph node as N1) (19). NPI scores were calculated according to Rampault et al (17), as follows: NPI = 0.2 × tumor size (cm) + lymph-node stage (1, 2, or 3) + histological grade (1, 2, or 3), where size was measured in centimeters; lymph node stage 1 was lymph node-negative, stage 2 was one to three positive lymph nodes, stage 3 was more than three positive lymph nodes; and the scoring of histological grade was 1 to 3 (see below). For prognostic considerations, NPI was categorized into three groups, as follows: low (good prognosis), NPI≤3.4; intermediate (moderate prognosis), 3.4<NPI≤5.4; and high (poor prognosis), NPI>5.4. Concentrations of estrogen and progesterone receptors in tumor cytosol were evaluated by the dextran-coated charcoal assay as described elsewhere (20). Concentration of estrogen receptors (ER) of ≥5 fmol/mg of protein and progesterone receptors (PR) of ≥10 fmol/mg of protein were considered to be positive (20).

Adjuvant therapies were based and prescribed according to the University Hospital Treatment Protocol for Breast Cancer (unpublished document for in-house use). Cut-off values for ER and PR at the University Hospital for Tumors (20) were less strict than usually recommended (10 fmol/mg of protein for ER and 20 fmol/mg of protein for PR) (21). Consequently, decisions about the adjuvant therapies were based on these lower cut-off values. Adjuvant radiotherapy consisted of external megavoltage irradiation delivered from the linear accelerator (22). Adjuvant chemotherapy included either the CMF protocol (cyclophosphamide, 600 mg/m2 IV on day 1; methotrexate, 40 mg/m2 IV on day 1; 5-fluorouracil, 600 mg/m2 IV on day 1) or the FAC protocol (5-fluorouracil, 500 mg/m2 IV on day

Table 1. Characteristics of 81 women diagnosed with invasive ductal breast cancer in 1995 after 5 years of follow-up

Characteristics*	No. of women with breast cancer				P†
	relapse-free (n = 23)	locoregional relapse (n = 30)	bone metastases (n = 28)	total	
Age at diagnosis (y):					
30-39	0	3	0	3	0.720‡
40-49	6	7	7	20	
50-59	7	7	7	21	
60-69	6	10	12	28	
70-79	3	2	1	6	
80-89	1	1	1	3	
Year of initial diagnosis	1995 (23)	1992 (1), 1993 (2), 1994 (8), 1995 (15), 1996 (2), 1997 (2)	1995 (9), 1997 (10), 1998 (7), 1999 (2)		
Type of operation:					
mastectomy with axillary dissection	20	30	26	76	0.143
segmentectomy with axillary dissection	3	0	2	5	
Disease relapse in 2000 (median, mo)	/	18	13		<0.001
range (mo)	/	(3-49)	(2-44)		
Tumor size (cm):					
<2	7	7	13	27	0.200
2-5	16	20	13	49	
>5	0	3	2	5	
Histological grade:§					
I	4	3	1	8	0.521
II	13	18	16	47	
III	6	9	11	26	
Axillary lymph node status:					
0	0	4	2	6	0.203
1-3	14	10	12	36	
≥4	9	16	14	39	
Nottingham Prognostic Index (NPI):					
≤3.4	1	3	1	5	0.383
3.41-5.4	14	11	16	41	
>5.4	8	16	11	35	
Estrogen receptor (fmol/mg):					
<5	10	22	21	53	0.032
≥5	13	8	7	28	
Progesterone receptor (fmol/mg):					
<10	8	15	8	31	0.225
≥10	15	15	20	50	
Adjuvant chemotherapy CMF/FAC	6/1	20/3	16/9	42/13	<0.001
Adjuvant tamoxifen	12	10	12	34	0.385
5-y survival:					
alive	23	7	2	32	<0.001
dead	–	18	22	40	
unknown	–	5	4	9	
10-y survival:					
alive	14	1	1	16	<0.001
dead	7	24	23	54	
unknown	2	5	4	11	

*Abbreviations: CMF – cyclophosphamide, methotrexate, 5-fluorouracil; FAC – 5-fluorouracil, doxorubicine, cyclophosphamide.
†Pearson χ^2 test for all values except time to disease relapse and 5-year survival (F test within analysis of variance test).
‡P value refers only to 40-69-year age groups, because other age groups had cell frequencies <5.
§Histological grading according to the method by Elston (18).

1; doxorubicine, 50 mg/m^2 IV on day 1; cyclophosphamide, 500 mg/m^2 IV on day 1). Cycles were repeated every 3 weeks, for a total of 6 cycles. Tamoxifen was administered as systemic hormonal therapy (2 × 10 mg over 5 years) (23).

Immunohistochemical analysis for MAGE-A4, NY-ESO-1, and HER-2

Immunohistochemical analysis of primary breast cancer tissue was performed in 2000. Expression of MAGE-A4 and NY-ESO-1 tumor-associated C/T antigens in primary breast cancer tissue was determined by monoclonal antibody (mAb) 57B (24) and mAb B9.8.1.1 (25), respectively. Within our panel of monoclonal antibodies, mAb 57B (23) recognized many MAGE-A-related gene products, including MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, and MAGE-A12 (26). In paraffin-embedded specimens, however, it had been shown to

predominantly recognize MAGE-A4 TAA (27). Monoclonal antibody B9.8.1.1 is specific to NY-ESO-1 TAA (25). Briefly, tissue sections from paraffin-embedded breast tumor samples (0.5 mm thick) were placed on Silane (3-aminopropyltriethoxysilane, A 3648, Sigma, St Louis, MO, USA)-treated microscope glass slides. After deparaffinization, the sections were heated in a 800W household microwave oven at a maximum power for 8.5 and 5 minutes in 10 mmol/L citric buffer (pH 6.0) and washed with phosphate buffered saline (PBS; pH 7.2). The sections were treated by H2O2 to suppress endogenous peroxidase activity. After an additional PBS wash, the sections were incubated for 20 minutes with 1:10 diluted normal rabbit sera (DAKO X0902, DAKO A/S, Copenhagen, Denmark) at room temperature in a humidified chamber to prevent nonspecific immunoglobulin binding. The sections were then treated with mAb 57B or mAb B9.8.1.1., in the form of undiluted hybridoma supernatant, for 90 minutes at room temperature. A streptoavidin-biotinylated horseradish peroxidase-based detection system (DAKO K 0355) was used to reveal specific binding (28,29).

Immunoreactivity for MAGE-A4 and NY-ESO-1 was scored in the following way: 0, no positive tumor cells (negative); 1+, <20% positive tumor cells ("mild reaction"); 2+, 21-50% positive tumor cells ("moderate reaction"); and 3+, >50% positive tumor cells ("strong reaction"). Non-neoplastic cells, such as normal ductal epithelial cells and fibroblasts, were indeed present in all specimens but were not stained, and thus served as internal negative controls (28,29). The immunoreactivity scores were presented as either "negative" or "positive," with positive including mild, moderate, and strong reactions.

DAKO Hercep TestTM kit was used for HER-2 immunohistochemical staining in accordance with manufacturer's instructions. The monoclonal antibody in the DAKO Hercep TestTM kit is approved by Food and Drug Administration as the specific reagent for the HER-2 detection. Samples with 3+ staining intensity score (standard control slides were included in the Hercep Test™ kit) were considered to be HER-2 positive. When Hercep test scoring system was used, a strong positive reaction implied a complete (diffuse) membrane staining in >10% of tumor cells (16).

Statistical analysis

STATISTICA 6.1 software package (StatSoft Inc., Tulsa, OK, USA) was used for all statistical analyses. Differences in time to disease relapse and 5-year survival were tested by one way analysis of variance (ANOVA). Pearson $\chi 2$ test was applied for other clinicopathologic and immunohistochemical parameters represented as qualitative values. Kaplan-Meier survival method was used for the construction of survival probability curves, and Gehan's Wilcoxon test for their comparison. P value of <0.05 was considered statistically significant.

Results

Most women were diagnosed with breast cancer in 1995 and most underwent modified radical mastectomy (Table 1). In the group of women with locoregional disease relapse, the median time to disease relapse was 18 months (range, 3-49), which was significantly longer than 13 months (range, 2-44) in the group with bone metastases (P<0.001). The three groups of patients did not differ in the standard clinical parameters except for ER status (P = 0.032) and adjuvant chemotherapy (P<0.001). In the 5-year relapse-free group, significantly more women had positive ER and significantly fewer received adjuvant chemotherapy; this group had a significantly better survival outcome (P<0.001). In 2005, 14 out of 23 women in this group were alive (with 2 women of unknown survival status), whereas in other two groups, only one women from each group was alive.

Immunohistochemical results

Immunohistochemical staining of C/T proteins, MAGE-A4 and NY-ESO-1, was predominantly visible as cytoplasmic staining limited to tumor cells (Figure 1A and 1B). HER-2 staining was visible as membrane staining (Figure 1C). Overall, positivity of mAb 57B and mAb B9.8.1.1 was found in 60 (74%) and 32 (40%) out of 81 women included in the study, respectively. The expression of MAGE-A4 antigen was detected in a significantly fewer women with locoregional relapse ($P = 0.014$; Table 2). Further analysis showed significant difference only in MAGE-A4 expression between the women with locoregional relapse and 5-year relapse-free group ($P = 0.005$). The expression of MAGE-A4 did not significantly differ between patients with locoregional relapse and those with bone metastases ($P = 0.076$). No significant difference was found in NY-ESO-1 antigen expression between the three groups of women. Positive HER-2 reaction was found in 18 out of 81 patients (22%), with equal distribution in all three groups.

Survival of patients was also analyzed with respect to the expression of MAGE-A4, NY-ESO-1, and HER-2 antigen. It was found that MAGE-A4-positive patients had a significantly better survival than MAGE-A4-negative patients ($P = 0.046$; Figure 2). In contrast, NY-ESO-1 and HER-2 antigen expression did not correlate with survival (data not shown).

Relation of MAGE-A4 antigen to the standard prognostic and predictive factors

Since the three groups of women with breast cancer significantly differed in MAGE-A4 antigen expression, we analyzed the relationship between MAGE-A4 antigen and standard prognostic and predictive factors (Table 3). MAGE-A4 antigen expression was found to be associated to a significant degree only with the NY-ESO-1 antigen expression ($P = 0.006$), but not with tumor size and grade, number of metastatically involved axillary lymph nodes, or ER and PR status. Such results suggest that in our patients, MAGE-A4 antigen might have behaved as a pro-

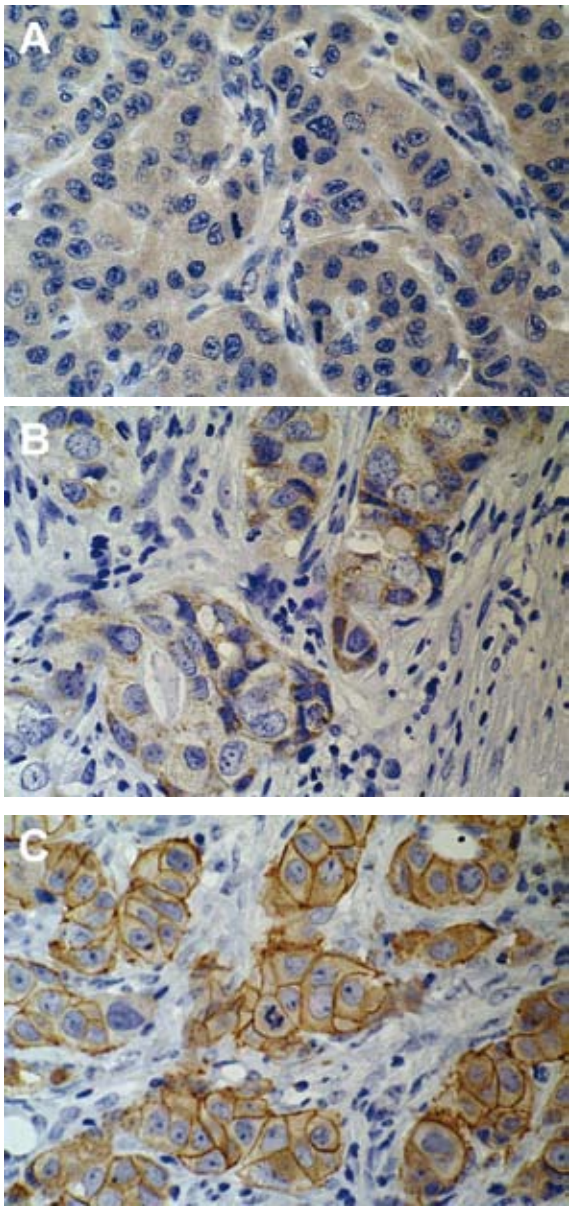

Figure 1. Immunohistochemical staining in invasive ductal breast cancer tissue. **A.** Intense cytoplasmic MAGE-A4 staining with monoclonal antibody (mAb) 57B observed in the absence of staining of normal ducts (peroxidase anti-peroxidase [PAP], ×400). **B.** NY-ESO-1 positivity with specific cytoplasmic tumor distribution detected by mAb B9.8.1.1 (PAP, ×400). **C.** Overexpression of HER-2 detected by Hercep test, with strong complete membrane staining in >10% of tumor cells, noticed as a strong immunohistochemical reaction (PAP, ×400).

Table 2. Immunohistochemically detected expression of HER-2, MAGE-A-4, and NY-ESO-1 antigens in 81 women with invasive ductal breast cancer

Antigen	No. (%) of women with breast cancer				*P**
	relapse-free for 5 y (n = 23)	locoregional relapse (n = 30)	bone metastases (n = 28)	total	
HER-2:					
negative (0, 1+, 2+)	19 (83.0)	23 (77.0)	22 (79.0)	64 (79.0)	0.868
positive (3+)	4 (17.0)	7 (23.0)	6 (21.0)	17 (21.0)	
MAGE-A4:†					
negative (0)	2 (9.0)	13 (43.0)	6 (21.0)	21 (26.0)	0.014‡
positive (1+, 2+, 3+)	21 (93.0)	17 (57.0)	22 (79.0)	60 (74.0)	
NY-ESO-1:†					
negative (0)	13 (57.0)	22 (73.0)	14 (50.0)	49 (60.0)	0.173
positive (1+, 2+, 3+)	10 (43.0)	8 (27.0)	14 (50.0)	32 (40.0)	

*Pearson χ^2 test.

†Immunoreactivity was scored in the following way: 0, no positive tumor cells (negative); 1+, ≤20% positive tumor cells ("mild reaction"); 2+, 21-50% positive tumor cells ("moderate reaction"); and 3+, >50% positive tumor cells ("strong reaction").

‡Comparison of data between locoregional group and 5-year relapse-free group (P = 0.005) and between locoregional group and group of patients with bone metastases (P = 0.076).

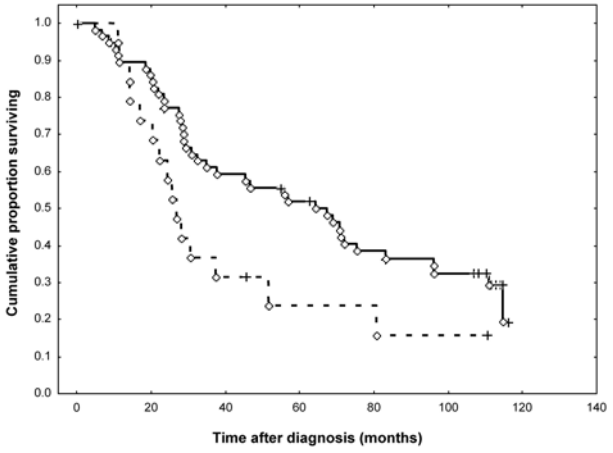

Figure 2. Overall survival of 81 women with invasive ductal breast cancer according to the MAGE-A4 expression. Full line – MAGE-A4 positive (1+, 2+, 3+) (n = 60); dotted line – MAGE-A4 negative (0) (n = 21); plus sign – censored data; circle – complete data. Gehan's Wilcoxon test, P = 0.046.

gnostic factor unrelated to the above standard prognostic and predictive factors.

Discussion

This retrospective study showed that the women with breast cancer who were disease-free for 5 years, those with locoregional relapse of the disease, and those with bone metastases differed in MAGE-A4 expression, but not in NY-ESO-1 and HER-2 expression. The three groups of women were comparable in clinicopathologic parameters, except for ER status and adjuvant systemic cytotoxic therapy. A significantly higher number of women with positive ER in the group of disease-free patients confirmed the positive prognostic and predictive role of ER. On the one hand, a significantly smaller number of women in this group received adjuvant chemotherapy, which was probably caused by higher proportion of ER positive cases, but on the other, there was no difference in adjuvant tamoxifen therapy between the three groups.

The biological course of the disease was probably not much influenced by the adjuvant chemotherapy and hormonal therapy in the women with locoregional relapse and those with bone metastatic disease. There were fewer women with positive MAGE-A4 antigen staining in the group with locoregional relapse than in the 5-year relapse-free group. No association was found between the MAGE-A4 immunohistochemical expression and standard prognostic and predictive markers. However, a link with the NY-ESO-1 immunohistochemical expression was found, which corresponds with previous findings of usually frequent concomitant expression of these two C/T antigens (13,30). Patients with positive and those with negative MAGE-A4 expression significantly differed in survival. Given the prognostic and predictive role of the HER-2 antigen, we would expect a similar difference in survival with respect to its expression (7,8,14), but it was

Table 3. Relation of MAGE-A4 expression detected by immunohistochemistry with tumor size, histological grade, axillary lymph node status, estrogen and progesteron receptor positivity, and HER-2 and NY-ESO-1 expression*

Characteristics	No. of women with breast cancer			
	MAGE-A4 negative (0)	MAGE-A4 positive (1+, 2+, 3+)	total	*P*†
Tumor size (cm):				
<2	8	19	27	0.601
2-5	11	38	49	
>5	2	3	5	
Histological grade:‡				
I	2	6	8	0.911
II	13	34	47	
III	6	20	26	
Axillary lymph nodes:				
negative	3	3	6	0.369
positive	18	57	75	
Estrogen receptors (fmol/mg):				
<5	17	36	53	0.082
≥5	4	24	28	
Progesterone receptors (fmol/mg):				
<10	10	21	31	0.306
≥10	11	39	50	
HER-2:				
negative (0, 1+, 2+)	19	44	63	0.134
positive (3+)	2	16	18	
NY-ESO-1:				
negative (0)	18	31	49	0.006
positive (1+, 2+, 3+)	3	*29*	*32*	

*Immunoreactivity for MAGE-A4 and NY.ESO-1 was scored in the following way: 0, no positive tumor cells (negative); 1+, ≤20% positive tumor cells ("mild reaction"); 2+, 21-50% positive tumor cells ("moderate reaction"); and 3+, >50% positive tumor cells ("strong reaction").
†Pearson χ^2 test.
‡Histological grading according to the method by Elston (18).

not detected. These results suggest that MAGE-A4, along with traditional prognostic factors, could have a prognostically independent relevance in patients with breast cancer.

Breast cancer biology is complex, with multiple factors contributing to the development and growth of cancer and its metastatic progression. Clinical data from follow-up studies and studies of the biology of breast cancer could be used to identify parameters that could serve as prognostic or predictive factors. Treatment decision making is usually based on a combination of clinical features and tumor characteristics, such as age, tumor size, histological type and grade, lymph node status, and ER and PR status (1-5). However, since the prognostic value of these criteria is variable, it is obvious that additional and still unidentified molecular factors influence and determine the clinical course of breast cancer. By identifying these additional factors, therapeutic approaches to patients with breast cancer could be further individualized, thus increasing both the survival rate and quality of life of the patients. Novel high-performance screening methods, such as the DNA microarray, which can analyze simultaneously the expressions of thousands of genes in a tissue in a single experiment, may allow the identification of disease subsets that correlate with clinical outcomes. Clearly, such gene-expression profiling (holistic approach), although not yet routinely used in clinical practice, would provide highly useful prognostic information (6-11).

Immunohistochemical detection of HER-2 antigen expression was used as the control for detection of MAGE-A4 and NY-ESO-1 expression. HER-2 molecule belongs to a family of four homologous receptors involved in the tyrosine kinase-mediated regulation of normal breast tissue growth and development. Overexpression of HER-2 molecule in breast cancer cells is associated with poor prognosis (7,8,16).

C/T TAA antigens were discovered in the 1990s, initially as targets in CD8 T-cell recognition of autologous human melanoma cells (31). To date, 44 C/T genes have been identified and their expression in numerous cancer types has been studied. The antigen is expressed in normal tissues, but seems to be restricted to testis, fetal ovary, and placenta, and in cancers of diverse origin. Up to 30-40% of many different cancer types, eg, melanoma, bladder cancer, and sarcoma, express one or more C/T antigens. X chromosome codes for the majority of C/T antigens; however, many recently defined C/T coding genes have non-X chromosomal loci. The function of most C/T antigens is unknown, although they seem likely to have some role in regulating gene expression. The global demethylation associated with certain cancers might account for the aberrant C/T expression in cancer. Another important issue is whether the expression of these

genes in the cancer cell contributes to its malignant behavior. There is increasing evidence that C/T expression is correlated with tumor progression and takes place in tumors of higher malignant potential (12,13,30).

The expression of C/T genes has mostly been studied on clinical material by use of polymerase chain reaction (PCR). However, PCR cannot show if the analyzed genes are expressed only in low percentage of tumor cells or in most of them. Therefore, clinical immunotherapy studies and trials should be aimed at antigens expressed in most – preferably all – tumor cells. Studies of therapeutic relevance are those where it is possible to quantify tumor cells expressing tumor antigens, such as immunohistochemical studies, which can be performed today because of the development of serological reagents (mAbs) against C/T TAA (13,30).

Expression of MAGE genes in breast cancers was reported by several groups (32-34), as well as particular MAGE-A1, -A2, -A3, -A4, -A6, and -A12 specific transcripts (35), whereas much less has been published on their immunodetection (30). Kavalar et al (28) reported a correlation between mAb 57B staining and the tumor grade, lymphatic vessel invasion, and intratumoral necrosis and an inverse correlation with ER staining.

Since this study was a preliminary investigation, the number of patients was relatively small, which may represent a limitation. Also, a small sample might be a possible reason why we did not observe the influence of HER-2 expression on patients' survival. Therefore, potential differences in unknown factors in our, relatively small, patient groups may have lead to biased results or conclusions. Accordingly, the observation that the MAGE-A4 antigen has a prognostic role is only an initial hypothesis that should be tested on a much greater number of patients with breast cancer.

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